

FORM PTO-1390 (Modified)
(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/913772INTERNATIONAL APPLICATION NO.
PCT/FR00/00393INTERNATIONAL FILING DATE
17 FEB 2000 (17.02.00)PRIORITY DATE CLAIMED
17 FEB 1999 (17.02.99)

TITLE OF INVENTION

**USE OF AN ENTEROBACTERIUM OmpA PROTEIN COMBINED WITH AN ANTIGEN, FOR
GENERATING AN ANTIVIRAL, ANTIPARASITIC, OR ANTITUMOR CYTOTOXIC RESPONSE**

APPLICANT(S) FOR DO/EO/US

Toufic RENNO and Jean-Yves BONNEFOY

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. has been transmitted by the International Bureau.
 - c. is not required, as the application was filed in the United States Receiving Office (RO/US).
6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. A copy of the International Search Report (PCT/ISA/210).
8. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. have been transmitted by the International Bureau.
 - c. have not been made; however, the time limit for making such amendments has NOT expired.
 - d. have not been made and will not be made.
9. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
11. A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. A **FIRST** preliminary amendment.
16. A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. A substitute specification.
18. A change of power of attorney and/or address letter.
19. Certificate of Mailing by Express Mail
20. Other items or information:

PTO 1449 listing references of International Search Report
Certificate of Mailing by Express Mail
Sequence Listing - Paper and Diskette
Statement under 37 CFR § 1.821(f)

U.S. APPLICATION NO. (IF KNOWN SEE 37 CFR
097913772INTERNATIONAL APPLICATION NO.
PCT/FR00/00393**531 Rec'd PCT/FR** 16 AUG 2001ATTORNEY'S DOCKET NUMBER
PE94 PCTSEO/dln

21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5) :

<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	\$1,000.00
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	\$860.00
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	\$710.00
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	\$690.00
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	\$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**\$860.00**Surcharge of **\$130.00** for furnishing the oath or declaration later than 20 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).**\$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	43 - 20 =	23	x \$18.00	\$414.00
Independent claims	2 - 3 =	0	x \$80.00	\$0.00
Multiple Dependent Claims (check if applicable).			<input type="checkbox"/>	\$0.00
			TOTAL OF ABOVE CALCULATIONS =	\$1,274.00
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).			<input type="checkbox"/>	\$0.00
			SUBTOTAL =	\$1,274.00
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).			<input type="checkbox"/> 20 <input type="checkbox"/> 30 +	\$0.00
				TOTAL NATIONAL FEE =
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).			<input type="checkbox"/>	\$0.00
			TOTAL FEES ENCLOSED =	\$1,274.00
			Amount to be: refunded	\$
			charged	\$

 A check in the amount of **\$1,274.00** to cover the above fees is enclosed. Please charge my Deposit Account No. in the amount of to cover the above fees. A duplicate copy of this sheet is enclosed. The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **8-3220** A duplicate copy of this sheet is enclosed.**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

**25666**

PATENT TRADEMARK OFFICE

G. Patrick Sage

SIGNATURE

G. Patrick Sage

NAME

37,710

REGISTRATION NUMBER

14 August 2001

DATE

09/913772

PF94PCTSEQ/dln

531 Rec'd PCT/... 16 AUG 2001

* * * * *

Applicant : Toufic Renno and Jean-Yves Bonnefoy

Title : USE OF AN ENTEROBACTERIUM OmpA PROTEIN
COMBINED WITH AN ANTIGEN, FOR GENERATING AN
ANTIVIRAL, ANTI PARASITIC OR ANTITUMOR CYTOTOXIC
RESPONSE

* * * * *

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

As soon as a Serial Number and Filing Date have been accorded the above-
identified national phase application, kindly amend as follows:

IN THE CLAIMS: Kindly cancel all of the claims and replace by Claims 44
through 86, attached.

IN THE ABSTRACT: Attached please find an Abstract of the Disclosure in U.S.
format.

R E M A R K S

The present application is a national phase filing of PCT/FR00/00393 of
February 17, 2000.

Applicants have cancelled all of the originally-filed Claims and replaced them with new Claims, 44 through 86, which better encompass the full scope and breadth of the invention notwithstanding Applicants belief that the Claims would have been allowable as originally filed. Accordingly, Applicants assert that no Claims have been narrowed within the meaning of *Festo*.

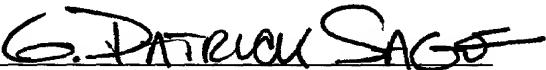
A U.S. format Abstract is provided.

A PTO 1449 listing all of the references cited in the International Search Report is provided.

Entry of the new Claims and Abstract and early and favorable action on the merits of this application are respectfully solicited.

Respectfully submitted,

THE FIRM OF HUESCHEN AND SAGE

By: 
G. PATRICK SAGE

Dated: August 9, 2001
Customer No.: 25,666
500 Columbia Plaza
350 East Michigan Ave.
Kalamazoo, MI 49007
(616) 382-0030

Enclosure: Postal Card Receipt
Sequence Listing - Paper copy
Sequence Listing - diskette copy
Statement under 37 CFR § 1.821(f)
Abstract of the Disclosure
Claims 44 through 86
PTO 1449

CLAIMS

- 44 -

The use of an enterobacterium OmpA protein, or of a fragment thereof, for preparing a pharmaceutical composition useful in generating or increasing a cytotoxic T response against an infectious agent or a tumor cell.

- 45 -

The use of Claim 44, wherein the pharmaceutical composition containing the enterobacterium OmpA protein, contains an antigen or a hapten specific for the infectious agent or for the tumor cell.

- 46 -

The use of Claim 44, wherein the infectious agent is a viral particle, a bacterium, or a parasite.

- 47 -

The use of Claim 44, wherein the enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of the enterobacterium.

- 48 -

The use of Claim 44, wherein the enterobacterium OmpA protein, or a fragment thereof, is obtained by recombination.

- 49 -

The use of Claim 44, wherein the enterobacterium is *Klebsiella pneumoniae*.

- 50 -

The use of Claim 49, wherein an amino acid sequence of the OmpA protein, or a fragment thereof, is selected from

- a) the amino acid sequence of SEQ ID No. 2;
- b) the amino acid sequence of a sequence having at least 80% homology with SEQ ID No. 2; and
- c) the amino acid sequence of a fragment of at least 5 amino acids of a sequence as defined in a).

- 51 -

The use of Claim 45, wherein the antigen or hapten is selected from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids and any compound capable of specifically directing a CTL response against an infectious agent or a tumor cell.

- 52 -

The use of Claim 45, wherein the antigen or hapten is coupled to or mixed with the OmpA protein or a fragment thereof.

- 53 -

The use of Claim 52, wherein the antigen or hapten is coupled, by covalent attachment, with the OmpA Protein or a fragment thereof.

- 54 -

The use of claim 53, wherein the coupling by covalent attachment is coupling produced by chemical synthesis.

- 55 -

The use of Claim 54, wherein one or more attachment elements is(are) introduced into the OmpA protein, or a fragment thereof, and/or into the antigen or hapten, in order to facilitate the chemical coupling.

- 56 -

The use of Claim 55, wherein the attachment element introduced is an amino acid.

- 57 -

The use of Claim 53, wherein the coupling between the antigen or hapten and the OmpA protein, or a fragment thereof, is produced by genetic recombination, wherein the antigen or hapten is a peptide in nature.

- 58 -

The use of Claim 57, wherein the pharmaceutical composition comprises a nucleic acid construct encoding the hybrid protein.

- 59 -

The use of Claim 58, wherein the nucleic acid construct is contained in a vector or in a transformed host cell capable of expressing the hybrid protein.

- 60 -

The use of Claim 44 for preparing a pharmaceutical composition intended to eliminate infectious agents or inhibit tumor growth.

- 61 -

The use of Claim 44 for preparing a pharmaceutical composition intended to prevent or treat infectious diseases comprising viral, bacterial, fungal and parasitic infections.

- 62 -

The use of Claim 44 for preparing a pharmaceutical composition intended to prevent or treat cancers.

- 63 -

The use of Claim 62 for preparing a pharmaceutical composition intended to prevent or treat cancers associated with a tumor antigen.

- 64 -

The use of Claim 62 for preparing a pharmaceutical composition intended to prevent melanomas.

- 65 -

The use of Claim 44, wherein the pharmaceutical composition is vehicled in a form making it possible to improve its stability and/or its immunogenicity.

- 66 -

The use of Claim 65, wherein the vehicle is selected from:

- a liposome,
- a viral vector containing a nucleic acid construct encoding the OmpA protein, a fragment thereof, an antigen or hapten, or a hybrid protein, and
- a transformed host cell capable of expressing the OmpA protein, a fragment thereof, an antigen or hapten, or a hybrid protein.

- 67 -

The use of Claim 58, wherein the nucleic acid construct or the nucleic acid construct contained in the vector or the transformed host cell

comprises a nucleic acid sequence chosen from SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides of SEQ ID No. 1, or a sequence having at least 80% homology with one of the sequences.

- 68 -

A pharmaceutical composition, containing at least one enterobacterium OmpA protein or a fragment thereof, combined by mixing or by coupling, with at least one antigen or one hapten associated with, or specific for, a tumor cell, in a pharmaceutically-acceptable medium.

- 69 -

The composition of Claim 68, wherein the enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of the enterobacterium.

- 70 -

The composition of Claim 68, wherein the enterobacterium OmpA protein, or a fragment thereof, is obtained by recombination.

- 71 -

The composition of Claim 68, wherein the enterobacterium is *Klebsiella pneumoniae*.

- 72 -

The composition of Claim 71, wherein the amino acid sequence of the OmpA protein, or a fragment thereof, is selected from:

- a) the amino acid sequence of SEQ ID No. 2;
- b) the amino acid sequence of a sequence having at least 80% homology with SEQ ID No. 2; and

c) the amino acid sequence of a fragment of at least 5 amino acids of a sequence as defined in a).

- 73 -

The composition of Claim 68, wherein the antigen or hapten is selected from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids and any compound capable of specifically directing a CTL response against the tumor cell.

- 74 -

The composition of Claim 68, wherein the antigen or hapten is coupled, by covalent attachment, with the OmpA protein or a fragment thereof.

- 75 -

The composition of Claim 74, wherein the coupling by covalent attachment is coupling produced by chemical synthesis.

- 76 -

The composition of Claim 75, wherein one or more attachment elements is(are) introduced into the OmpA protein, or a fragment thereof, and/or into the antigen or hapten, in order to facilitate the chemical coupling.

- 77 -

The composition of Claim 76, wherein the attachment element introduced is an amino acid.

- 78 -

The composition of Claim 74, wherein the coupling between the antigen or hapten and the OmpA protein, or a fragment thereof, is produced by genetic recombination, wherein the antigen or hapten is a peptide in nature.

- 79 -

The composition of Claim 75, wherein the pharmaceutical composition comprises a nucleic acid construct encoding the hybrid protein obtained after the coupling.

- 80 -

The composition of Claim 79, wherein the nucleic acid construct is contained in a vector or in a transformed host cell capable of expressing the hybrid protein.

- 81 -

The composition of Claim 79, wherein the nucleic acid construct comprises a nucleic acid sequence chosen from SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides of SEQ ID No. 1, or a sequence having at least 80% homology with SEQ ID No. 1.

- 82 -

The composition of Claim 68, wherein the pharmaceutical composition is vehicled in a form which makes it possible to improve its stability and/or its immunogenicity.

- 83 -

The composition of Claim 82, wherein the vehicle is selected from:

- a liposome,
- a viral vector containing a nucleic acid construct encoding the OmpA protein, a fragment thereof, an antigen or hapten, or a hybrid protein, and
- a transformed host cell capable of expressing the OmpA protein, a fragment thereof, an antigen or hapten, or a hybrid protein.

- 84 -

The composition of Claim 68, wherein the pharmaceutically-acceptable medium consists of water, an aqueous saline solution, or an aqueous solution based on dextrose and/or on glycerol.

- 85 -

The composition of Claim 68, wherein the composition also contains a detergent.

- 86 -

The composition of Claim 68, without any other adjuvant for inducing a CTL response.

PROCESSED IN ACCORDANCE WITH 37 CFR 1.56

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :

U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR00/00393

VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the French language in which the below identified international application was filed, and that, to the best of my knowledge and belief, the English translation of the international application No. PCT/FR00/00393 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: July 26, 2001



Full name of the translator : Elaine Patricia PARRISH

For and on behalf of RWS Group plc

Post Office Address : Europa House, Marsham Way,
Gerrards Cross, Buckinghamshire,
England.

ONLY FOR INFORMATION

Codes used to identify the PCT member States on the flyleaves of the brochures in which international applications made under the PCT are published.

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531 Rec'd PCT/ 16 AUG 2001

USE OF AN ENTEROBACTERIUM OmpA PROTEIN COMBINED WITH AN ANTIGEN, FOR GENERATING AN ANTIVIRAL, ANTIPARASITIC OR ANTITUMOR CYTOTOXIC RESPONSE

5 The invention relates to the use of an enterobacterium, in particular *Klebsiella pneumoniae*, OmpA membrane protein, combined with an antigen or a hapten, for preparing a pharmaceutical composition intended to generate or increase a cytotoxic T response directed
10 against an infectious agent or a tumor cell. The invention comprises the use of these compounds for preventing and treating infection or cancer, in particular cancers combined with a tumor antigen, such as melanomas, and also for pharmaceutical compositions
15 comprising some of these compounds.

Immunization is an effective means of preventing or reducing viral or bacterial infections. The success of immunization campaigns in these domains has made it
20 possible to extend the vaccine concept, until now used in the domain of infectology, to the domains of cancer and of autoimmune diseases. Immunization antigens administered alone to the host are often not immunogenic enough to induce an immune response and
25 must, therefore, be combined with an adjuvant or coupled to a carrier protein in order to induce (or increase) the immunogenicity. Under these conditions, only an immune response of the humoral type can be induced. Now, in the context of antiviral therapy, the
30 generation of cytotoxic T lymphocytes (CTLs) capable of recognizing and destroying the virus is of great importance (Bachmann et al., 1994, Eur. J. Immunol., 24, 2228-2236; Borrow P., 1997, J. Virol. Hepat., 4, 16-24), as attested by many studies showing, *in vivo*,
35 the protective role of responses directed against viral epitopes (Arvin AM, 1992, J. Inf. Dis., 166, S 35-S41; Koszinowski et al., 1987 Immunol. Lett., 16, 185-192).

The importance of CTL responses has also been greatly documented in antitumor responses, in particular those directed against melanoma cells (review in Rivoltini et al., 1998, Crit. Rev. Immunol. 18, 55-63). The CTL epitope(s) (peptide sequences which interact with class I molecules and are presented to CD8+ T lymphocytes) have been defined for several antigens. However, the difficulty lies in generating CTLs *in vivo*, due to the weak immunogenicity of these peptides (Melialf, 1992, 5 Adv. Cancer Res., 58, 143-175; Nandaz and Sercaz, 1995, Cell, 82, 13-17).

Research is consequently directed toward identifying novel adjuvants, or an antigen delivery system, making 10 it possible to induce CTLs. Due to their effectiveness in presenting antigens and in stimulating the immune system, dendritic cells, for example, have been used to generate antiviral CTL responses (Ludewig B et al., 1998, J. Virol., 72, 3812-3818; Brossard P. et al., 15 1997, J. Immunol., 158, 3270-3276) or anticancer CTL responses (Nestle F.O. et al., 1998, Nat. Med., 4, 328-332). The approaches have consisted in loading the dendritic cells *ex vivo*, with the antigen of interest (peptides or cell lysate) and reimplanting these cells 20 into the patient. Other approaches consist in transfecting, *ex vivo*, the dendritic cells with the gene encoding the antigen of interest and in reinjecting these transfected cells (Gilboa E. et al., 1998, Cancer Immunol. Immunother., 46, 82-87). These 25 approaches have been used successfully in mice and recently in humans (Hsu F.J. et al., 1996, Nat. Med., 2, 52-58), but nevertheless remain complex since the cells must be treated *ex vivo* (transformation of the cells or internalization of the antigens) and 30 transplanted into the host organism. Similarly, the use of viral-type particles (Layton G.T. et al., 1993, J. Immunol., 151, 1097-1107) or of incomplete Freund's adjuvant (IFA) (Valmori et al., Eur. J. Immunol., 1994, 35 24, 1458-1462) makes it possible to generate CTL

responses. However, antiviral or antitumor immunization carried out with peptides corresponding to CTL epitopes and in the presence of such an adjuvant may lead to a state of specific tolerance, which may, in certain 5 cases, produce the opposite effect to that desired, i.e. a decrease in the immune response (Toes et al., Proc. Nat. Acad. Sci. USA, 1996, 93, 7855-7860).

Thus, there exists, today, a great need for a compound 10 which, when combined with a molecule, in particular an antigen or hapten, is capable of generating CTLs directed against said molecule. Such a compound could, in particular, be used for preparing an immunization composition intended to induce immune protection of the 15 antiviral, antibacterial, antifungal, antiparasitic or antitumor CTL type.

Surprisingly, it has been demonstrated that an outer membrane protein of a gram-negative bacterium, in 20 particular an enterobacterium OmpA protein such as the *Klebsiella pneumoniae* P40 protein (protein described in WO 95/27787 and WO 96/14415), has the property of eliciting a CTL response against a molecule which is covalently or noncovalently associated with it, 25 preferably without having to add another adjuvant.

Thus, the present invention relates to the use of an enterobacterium OmpA protein, of a fragment thereof or of a nucleic acid sequence encoding said OmpA protein 30 or a fragment thereof, for preparing a pharmaceutical composition intended to generate or increase a cytotoxic T response against an infectious agent or a tumor cell, *in vitro* or *in vivo*, preferably *in vivo*, and also for preparing a pharmaceutical composition 35 intended to generate or increase said cytotoxic T response.

In the present invention, the term "protein" is intended to denote both peptides or polypeptides and

the term "OmpA" (for "outer membrane protein") is intended to denote outer membrane proteins of the A type.

5 The expression "fragment of an OmpA protein" is intended to denote, in particular, any fragment of amino acid sequence included in the amino acid sequence of the OmpA protein which, when it is combined with an antigen or hapten specific for an infectious agent or
10 for a tumor cell, is capable of generating or increasing a cytotoxic T response directed against said infectious agent or said tumor cell, said fragment of the OmpA protein comprising at least 5 amino acids, preferably at least 10 amino acids or more preferably
15 at least 15 amino acids.

The expression "antigen or hapten specific for an infectious agent or for a tumor cell" is intended to denote, in particular, any compound expressed by an
20 infectious agent, such as a virus, a bacterium, a yeast, a fungus or a parasite, or by a tumor cell, or a structural analog thereof, which, alone or in combination with an adjuvant of immunity, is capable of inducing an immune response specific for said
25 infectious agent or for said tumor cell.

In the present description, the expression "analog of an antigen or hapten" is intended to denote, in particular, a compound having structural similarity
30 with said antigen or hapten, capable of inducing an immunological response directed against said antigen or hapten in an organism immunized beforehand with said similar compound.

35 A subject of the invention is also the use as claimed in the invention, characterized in that said pharmaceutical composition also comprises, combined with said enterobacterium OmpA protein, an antigen or a

hapten specific for said infectious agent or for said tumor cell.

Preferably, the invention comprises the use as claimed
5 in the invention, characterized in that said infectious agent is a viral particle, a bacterium, a yeast, a fungus or a parasite.

In a particular embodiment, the invention comprises the
10 use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said
15 enterobacterium.

The methods for extracting bacterial membrane proteins are known to those skilled in the art and will not be developed in the present description. Mention may, for
20 example, be made, but without being limited thereto, of the extraction method described by Haeuw J.H. et al. (Eur. J. Biochem, 255, 446-454, 1998).

In another preferred embodiment, the invention also
25 comprises the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained via the recombinant route.

30 The methods for preparing the recombinant proteins are, today, well known to those skilled in the art and will not be developed in the present description; reference may however be made to the method described in the examples. Among the cells which may be used for producing these recombinant proteins, mention should, of course, be made of bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology

4:520-525), and also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures
5 (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4:558-563), and also insect cells in which methods may be used which implement, for example, baculoviruses (Luckow V.A., 1993, Baculovirus systems
10 for the expression of human gene products. Curr. Op. Biotechnology 4:564-572).

Entirely preferably, the use as claimed in the invention is characterized in that said enterobacterium
15 is *Klebsiella pneumoniae*.

In particular, the invention relates to the use as claimed in the invention, characterized in that the amino acid sequence of said *Klebsiella pneumoniae* OmpA
20 protein, or a fragment thereof, comprises:

- a) the amino acid sequence of sequence SEQ ID No. 2;
- b) the amino acid sequence of a sequence having at least 80%, preferably 90% and 95% homology, after optimal alignment, with the sequence SEQ ID No. 2;
25 or
- c) the amino acid sequence of a fragment of at least 5 amino acids, preferably 10, 15, 20 and 25 amino acids, of a sequence as defined in a).

30 The expression "nucleic acid or amino acid sequence having at least 80% homology, after optimal alignment, with a given nucleic acid or amino acid sequence" is intended to denote a sequence which, after optimal alignment with said given sequence, comprises a percentage identity of at least 80% with said given sequence.

For the purposes of the present invention, the term "percentage identity" between two nucleic acid or amino acid sequences is intended to denote the percentage of nucleotides or of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two nucleic acid or amino acid sequences are conventionally carried out by comparing these sequences after having aligned them optimally, said comparison being carried out by segment or by "window of comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, other than manually, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], or by means of the local homology algorithm of Neddleman and Wunsch (1970) [J. Mol. Biol. 48:443], or by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], or by means of computer software which uses these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or with BLAST N or BLAST P comparison software).

The percentage identity between two nucleic acid or amino acid sequences is determined by comparing these two sequences which are optimally aligned by the window of comparison in which the region of the nucleic acid or amino acid sequence to be compared may comprise additions or deletions with respect to the reference sequence for optimal alignment between these two sequences. The percentage identity is calculated by determining the number of identical positions for which the nucleotide or the amino acid residue is identical between the two sequences, dividing this number of identical positions by the total number of positions in

the window of comparison and multiplying the result obtained by 100 in order to obtain the percentage identity between these two sequences.

5 Use may, for example, be made of the BLAST program "BLAST 2 sequences", which is available on the site <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>, the parameters used being those given by default (in particular for the "open gap penalty" parameter: 5, and
10 the "extension gap penalty" parameter: 2; the matrix chosen being, for example, the "BLOSUM 62" matrix provided by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

15 Among said sequences having at least 80% homology with the reference OmpA sequence, preference is given to the sequences of, or encoding, peptides capable of inducing CTL activity directed specifically against the antigen
20 or hapten which is combined with it, such as the CTL activity measured using the standard techniques described in the examples hereinafter.

25 The invention also comprises the use as claimed in the invention, characterized in that said antigen or hapten is chosen from proteins, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing the CTL response against said infectious agent or said tumor cell.

30 A subject of the present invention is also the use as claimed in the invention, characterized in that said antigen or hapten is coupled to or mixed with said OmpA protein or a fragment thereof.

35 The invention also comprises the use as claimed in the invention, characterized in that said antigen or hapten is coupled by covalent attachment, in particular by

chemical coupling, with said OmpA protein or a fragment thereof.

In a particular embodiment, the use as claimed in the invention is characterized in that one or more attachment elements is(are) introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate the chemical coupling; preferably, said attachment element introduced is an amino acid.

10

As claimed in the invention, it is possible to introduce one or more attachment elements, in particular amino acids, in order to facilitate the coupling reactions between the OmpA protein, or a fragment thereof, and said antigen or hapten. The covalent coupling between the OmpA protein, or a fragment thereof, and said antigen or hapten as claimed in the invention may be carried out at the N- or C-terminal end of the OmpA protein or a fragment thereof. The difunctional reagents which enable this coupling will be determined as a function of the end of the OmpA protein, or a fragment thereof, which is chosen for carrying out the coupling, and of the nature of said antigen or hapten to be coupled.

25

In another particular embodiment, the use as claimed in the invention is characterized in that the coupling between said antigen or hapten and said OmpA protein, or a fragment thereof, is produced by genetic recombination, when said antigen or hapten is peptide in nature.

The conjugates derived from coupling to said OmpA protein, or a fragment thereof, may be prepared by genetic recombination. The chimeric or hybrid protein (conjugate) may be produced using recombinant DNA techniques, by inserting or adding a sequence encoding said antigen or hapten which is peptide in nature into

the DNA sequence encoding said OmpA protein or a fragment thereof.

The methods for synthesizing the hybrid molecules
5 encompass the methods used in genetic engineering for constructing hybrid polynucleotides encoding desired polypeptide sequences. Advantageously, reference may, for example, be made to the technique for obtaining genes encoding fusion proteins, described by
10 D.V. Goeddel (Gene expression technology, Methods in Enzymology, Vol. 185, 3-187, 1990).

In another aspect, the invention relates to the use as claimed in the invention, characterized in that the pharmaceutical composition comprises a nucleic acid construct encoding said hybrid protein, or comprises a vector containing a nucleic acid construct encoding said hybrid protein or a transformed host cell containing said nucleic acid construct, which is
20 capable of expressing said hybrid protein.

The invention also comprises the use as claimed in the invention, for preparing a pharmaceutical composition intended to eliminate infectious agents or inhibit
25 tumor growth.

Preferably, the use as claimed in the invention relates to the preparation of a pharmaceutical composition intended to prevent or treat infectious diseases or
30 cancers, preferably cancers associated with a tumor antigen.

Among cancers in which the tumors express an associated tumor antigen, and which may be prevented or treated
35 with the uses as claimed in the present invention, mention may be made, in particular, but without being limited thereto, of:

- breast cancer, lung cancer, colon cancer and gastric carcinoma (Kawashima et al., 1999, Cancer Res. 59:431-5);
- mesothelioma, osteosarcoma, brain cancers (Xie 5 et al., 1999, J. Natl. Cancer Inst. 91:169-75);
- melanoma (Zheuten et al., 1998, Bratilsl. Lek. Listy 99:426-34);
- cystic adenoma of the pancreas (Hammel et al., 1998, Eur. J. gastroenterol. Hepatol. 10:345-8);
- 10 • colorectal cancer (Ogura et al., 1998, Anticancer Res. 18:3669-75);
- renal cell carcinoma (Jantzer et al., 1998, Cancer Res. 58:3078-86); and
- cancer of the ovary and of the cervix (Sonoda et al., 15 1996, Cancer. 77:1501-9).

A subject of the invention is in particular the use of an enterobacterium OmpA protein, or of a fragment thereof, as claimed in the invention, for preparing a pharmaceutical immunization composition intended to prevent or treat an infectious disease, in particular of viral, bacterial, fungal or parasitic origin, or a cancer, preferably associated with a tumor antigen, in particular melanomas.

25 The invention also comprises the use as claimed in the invention, characterized in that said pharmaceutical composition is vehicled in a form which makes it possible to improve its stability and/or its 30 immunogenicity, in particular in the form of a liposome.

Preferably, the invention comprises the use as claimed in the invention, characterized in that said vehicle is a viral vector containing a nucleic acid construct 35 encoding said OmpA protein or a fragment thereof, said antigen or hapten, or said hybrid protein, or a transformed host cell capable of expressing said OmpA

protein or a fragment thereof, said antigen or hapten, or said hybrid protein.

The invention also comprises the use as claimed in the
5 invention, characterized in that said nucleic acid construct, or the nucleic acid construct contained in said vector or said transformed host cell, comprises a nucleic acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15
10 consecutive nucleotides, preferably 20, 25, 30, 40 and 50 consecutive nucleotides, of the sequence SEQ ID No. 1, or a sequence having at least 80%, preferably 90% and 95%, homology, after optimal alignment, with one of said sequences.

15

In another aspect, the invention comprises a pharmaceutical composition as defined above in the uses as claimed in the present invention.

20 Among these compositions, preference is given to the pharmaceutical compositions characterized in that they comprise, in a pharmaceutically acceptable medium, at least one enterobacterium OmpA protein, or a fragment thereof, combined, by mixing or by coupling, with at
25 least one antigen or one hapten associated with or specific for a tumor cell.

For the purposes of the present invention, the pharmaceutically acceptable medium is the medium in
30 which the compounds of the invention are administered, preferably a medium which can be injected into humans. It may consist of water, of an aqueous saline solution or of an aqueous solution based on dextrose and/or on glycerol.

35

In one particular embodiment, the composition as claimed in the invention also contains a detergent.

The compositions as claimed in the invention may also contain a detergent, and in particular any type of pharmaceutically acceptable surfactant, such as for example anionic, cationic, nonionic or amphoteric 5 surfactants. Use is preferably made of the detergents Zwittergent 3-12 and octylglucopyranoside, and even more preferably Zwittergent 3-14.

Preferably, the pharmaceutical composition as claimed 10 in the invention is characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said enterobacterium or via the recombinant route.

15 Again preferably, the pharmaceutical composition as claimed in the invention is characterized in that said enterobacterium is Klebsiella pneumoniae.

In a preferred embodiment, the invention relates to a 20 composition as claimed in the invention, characterized in that the aminoacid sequence of said OmpA protein, or a fragment thereof, comprises:

- a) the amino acid sequence of sequence SEQ ID No. 2;
- b) the amino acid sequence of a sequence having at 25 least 80% homology with the sequence SEQ ID No. 2; or
- c) the amino acid sequence of a fragment of at least 5 amino acids, preferably 10, 15, 20 and 25 amino acids, of a sequence as defined in a).

30 Among the antigens or haptens which are part of the composition as claimed in the invention, preference is give to those chosen from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, 35 lipids or any compound capable of specifically directing a CTL response against a tumor cell.

In an equally preferred embodiment, the invention relates to a composition as claimed in the invention,

characterized in that said antigen or hapten is coupled, by covalent attachment, with said OmpA protein, or a fragment thereof, in particular by coupling produced by chemical synthesis and for which, 5 where appropriate, one or more attachment elements, such as an amino acid, may be introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate said chemical coupling.

10

In an equally preferred embodiment, the invention relates to a composition as claimed in the invention, characterized in that the coupling between said antigen or hapten and said OmpA protein, or a fragment thereof, 15 is produced by genetic recombination, when said antigen or hapten is peptide in nature (expression of a hybrid protein).

Thus, the present invention also relates to a 20 composition as claimed in the invention, characterized in that the pharmaceutical composition comprises a nucleic acid construct encoding said hybrid protein, said nucleic acid construct possibly being contained in a vector, or in a transformed host cell capable of 25 expressing said hybrid protein.

In a preferred embodiment, the invention relates to a composition as claimed in the invention, characterized in that said nucleic acid construct comprises a nucleic 30 acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides of the sequence SEQ ID No. 1, or a sequence having at least 80% homology with one of said sequences.

35

Among the compositions as claimed in the invention, preference is also given to the pharmaceutical compositions vehicled in a form which makes it possible to improve their stability and/or their immunogenicity,

in particular in the form of a liposome, of a viral vector containing a nucleic acid construct encoding said OmpA protein, or a fragment thereof, said antigen or hapten, or said hybrid protein, of of a transformed 5 host cell capable of expressing said OmpA protein, or a fragment thereof, said antigen or hapten, or said hybrid protein.

In a final aspect, the composition as claimed in the 10 invention is characterized in that it contains no other adjuvant for inducing a CTL response, besides said enterobacterium OmpA protein, or a fragment thereof, or a nucleic acid construct encoding said OmpA protein, or a fragment thereof, a characteristic element of the 15 composition as claimed in the invention for inducing a CTL response.

The legends to the figures and examples which follow 20 are intended to illustrate the invention without in any way limiting the scope thereof.

Legends to the figures:

Figures 1A, 1B, 1C and 1D: Measurement of the anti-MELAN-A and anti-TRP-2 CTL activity of effector cells

After immunization with 50 µg of hELA mixed with 3 µg 25 of rP40 (figure 1A), 50 µg of hELA mixed with 300 µg of rP40 (figure 1B), 50 µg of hELA coupled to rP40 30 (figure 1C) or 50 µg of the TRP-2 peptide mixed with 300 µg of rP40 (figure 1D), the draining lymph mode cells are stimulated with EL-4 A2/Kb cells (figures 1A, 1B and 1C) or EL-4 cells (figure 1D) which had been irradiated and prepulsed with 1 µM of the relevant 35 peptide, before being evaluated for their capacity to kill target cells which may (rectangle) or may not (diamond) have been prepulsed with the relevant peptide.

The X-axes of the points of figures 1A to 1D correspond to the ratio of the effector T cells (active lymphocytes) mixed together with the target cells (EL-4 A2/Kb or EL-4).

5

Figures 2A, 2B, 2C and 2D: Measurement of the anti-MELAN-A CTL activity of effector cells in the presence of the rP40 protein compared to the CTL activity obtained with standard immunization protocol.

10

After immunization with hELA (50 µg) alone (ELA, figure 2A), hELA mixed with 300 µg of rP40 (ELA + P40, figure 2B), hELA coupled to 300 µg of rP40 (ELA/P40, figure 2C) or hELA mixed with 50 µg of P30 peptide adjuvanted with IFA (ELA + IFA + TT, figure 2D) (IFA for Incomplete Freund's Adjuvant and TT for Tetanus Toxoid), the draining lymph node cells are stimulated in vitro for two weeks with EL-4 A2/Kb cells which have been irradiated and prepulsed with 1 µM of the relevant peptide, before being evaluated for their capacity to kill EL-4 A2/Kb target cells which may (rectangle) or may not (triangle) have been prepulsed with the hELA peptide.

Figures 3A, 3B, 3C and 3D: CTL activity and antitumor effect of the immunization with rP40 + TRP-2 peptide.

Figure 3A: The immunization with a mixture of the TRP-2 peptide with rP40 induces a CTL response specific for the peptide. C57BL/6 mice were injected subcutaneously with 50 µg of the TRP-2 peptide mixed with 300 µg of rP40. Ten days later, the lymph nodes were dissociated and restimulated with irradiated EL-4 cells which may (rectangles) or may not (diamonds) have been pulsed with the TRP-2 peptide.

35

Figures 3B, 3C and 3D: C57BL/6 mice received 2×10^3 cells of the B16F10 autologous melanoma, subcutaneously into the flank. Simultaneously (figures 3B and 3D), or 4 days later (figure 3C), some of these mice were

immunized subcutaneously (at the base of the tail) with 50 µg of the TRP-2 peptide mixed with 300 µg of rP40 (○), and others were immunized with the P40 protein alone (■, for figures 3B and 3C) or with the TRP-2 peptide alone (■, for figure 3D). From the 18th day postimplantation, the volume of the tumors was measured.

Figures 4A, 4B and 4C: Measurement of the anti-OVA CTL activity after immunization with the rP40 protein coupled to the p257-264 OVA peptide.

C57BL/6 mice received, by subcutaneous injection at the base of the tail, 200 µg of P40-Ova (■), of Ova-coupled beads (○), of solubilized Ova (□), of Ova-BS³ (▲) (BS³ for bis(succinimidyl) suberate), of P40 (←), or of DT-Ova (●) (DT for diphtheria toxoid).

The EL4 thymoma target cells pulsed with 50 µg/ml of OVA peptide (figure 4B) or not pulsed (figure 4C), or transfected with the ova gene (E.G7 line) (figure 4A) are incubated with ⁵¹Cr at 37°C and cultured with the effector cells.

Example 1: Cloning of the gene encoding the *Klebsiella pneumoniae* P40 protein

The gene encoding the P40 protein was obtained by PCR amplification using the genomic DNA of *Klebsiella pneumoniae* IP I145 (Nguyen et col., Gene, 1998). The gene fragment encoding this gene is inserted into various expression vectors under the control of various promoters, in particular that of the Trp operon. The nucleotide sequence and the peptide sequence of the P40 protein are represented by the sequences SEQ ID No. 1 and SEQ ID No. 2 hereinafter. An *E. coli* K12 producer strain was transformed with a pvaLP40 expression vector. The recombinant P40 protein (named rP40) is produced, in the form of inclusion bodies, with a

considerable yield (> 10% in g of protein/g of dry biomass).

This example is merely an illustration of the
5 expression of the rP40 protein, this illustration
possible being extended to other bacterial strains and
to other expression vectors.

10 Example 2: Method for fermentation of rP40 fusion
proteins

An Erlenmeyer flask containing 250 ml of TSB (Tryptic
Soy Broth, Difco) medium containing ampicilline
(100 µg/ml, Sigma) and tetracyclin (8 µg/ml, Sigma) is
15 inoculated with the transformed *E. coli* strain
described above. After overnight incubation at 37°C,
200 ml of this culture are used to seed 2 liters of
culture medium in a fermenter (Biolaffite, France). In
20 a quite conventional way, the culture medium may be
composed of chemical agents supplemented with vitamins
and/or yeast extracts, which are known to promote high
density bacterial cell growth.

The parameters controlled during the fermentation are:
25 pH, stirring, temperature, level of oxygenation and
supply of combined sources (glycerol or glucose). In
general, the pH is regulated at 7.0 and the temperature
is fixed at 37°C. The growth is controlled by supplying
glycerol (87%) at a constant rate (12 ml/h) in order to
30 maintain the dissolved oxygen tension signal at 30%.
When the turbidity of the culture (measured at 580 nm)
reaches the value of 80 (after culturing for
approximately 24 hours), the protein production is
treated by adding indole acrylic acid (IAA) at the
35 final concentration of 25 mg/l. Approximately 4 hours
after induction, the cells are harvested by
centrifugation. The amount of wet biomass obtained is
approximately 200 g.

Example 3: Method for extracting and purifying the rP40 protein

Extracting the rP40

5 After centrifugation of the culture broth (4000 rpm (revolutions per minute), 10 min, 4°C), the cells are resuspended in a 25 mM Tris-HCl buffer, pH 8.5. The insoluble components, or inclusion bodies, are obtained after treatment with lysozyme (0.5 g/liter, 1 hour at
10 room temperature with gentle stirring). The inclusion body pellet obtained by centrifugation (15 min at 10,000 g at 4°C) is taken up in a 25 mM Tris-HCl buffer at pH 8.5 containing 5 mM MgCl₂ and then centrifuged (15 min at 10,000 g).

15 The inclusion bodies are solubilized at 37°C for 2 hours in a 25 mM Tris-HCl buffer, pH 8.5, containing 7 M urea (denaturing agent) and 10 mM of dithiothreitol (reduction of disulfide bridges). Centrifugation
20 (15 min at 10,000 g) makes it possible to eliminate the insoluble particles.

This is then followed by resuspension in 13 volumes of 25 mM Tris-HCl buffer, pH 8.5, containing NaCl
25 (8.76 g/l) and Zwittergent 3-14 (0.1%, w/v). The solution is left overnight at room temperature with gentle stirring in contact with the air (to promote renaturation of the protein by dilution and reoxidation of the disulfide bridges).

30 Purifying the rP40 protein

- Anion exchange chromatography step

35 After a further centrifugation, the solution is dialyzed against a 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% Zwittergent 3-14 (100 volumes of buffer) overnight at 4°C.

The dialyzate is loaded on to a column containing a support of the strong anion exchanger type (Biorad Macro Prep High Q gel) equilibrated in the buffer described above, at a linear flow rate of 15 cm/h. The 5 proteins are detected at 280 nm. The rP40 protein is eluted, with a linear flow rate of 60 cm/h, for an NaCl concentration of 0.2 M in the 25 mM Tris-HCl buffer, pH 8.5: 0.1% Zwittergent 3-14.

10 - Cation exchange chromatography step

The fractions containing the rP40 protein are pooled and concentrated by ultrafiltration with the aid of an Amicon cell system with stirring, used with a YM10-type 15 Diaflo membrane (10 kDa cut-off threshold), for volumes of about 100 ml, or with the aid of a Millipore Minitan tangential flow filtration system, used with membrane plates having a 10 kDa cut-off threshold, for larger volumes. The fraction thus concentrated is dialyzed 20 overnight at 4°C against a 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14.

The dialyzate is loaded on to a column containing a support of the strong cation exchanger type (Biorad Macro Prep High S gel) equilibrated in the 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 25 3-14. The rP40 protein is eluted (rate 61 cm/h) for a 0.7 M NaCl concentration. The electrophoretic profiles show about a 95% degree of purity. The condition of the 30 protein is monitored by SDS-PAGE. The P40 protein, extracted from the *Klebsiella pneumoniae* membrane, has a characteristic electrophoretic (migration) behavior depending on whether it is in denatured or native form. The native form (β -sheet structure) in fact has a lower 35 molecular mass than the form which is denatured (α -helical structure) by the action of a denaturing agent, such as urea or guanidine hydrochloride, or by heating at 100°C in the presence of SDS. The rP40 protein is not properly renatured at the end of renaturation,

regardless of whether the latter is carried out in the presence or absence of 0.1% (w/v) Zwittergent 3-14. On the other hand, total renaturation is obtained after dialysis against a 25 mM Tris/HCl buffer, pH 8.5,
5 containing 0.1% (w/v) Zwittergent 3-14. However, it should be noted that this renaturation is only obtained when the dilution step and treatment at room temperature are, themselves, carried out in the presence of Zwittergent 3-14 (negative results in the
10 absence of detergent).

Example 4: Generation of CTLs

The antitumor CTL responses directed against melanoma
15 cells were defined for several antigens. These antigens are included in one of three categories:

- a) rejection antigen specific for melanoma, such as those of the MAGE family (review by van der Bruggen et
20 al., Science 254:1643);
- b) antigens resulting from the mutation of normal proteins. This group includes MUM-1 (Coulie et al., Proc. Natl. Acad. Sci. USA 92:7976-7980 (1995)); CDK4 (Wolfel et al., Science 296:1281-1284 (1995)) and HLA-
25 A2 (Brandel et al., J. Exp. Med. 183:2501-2508 (1996));
- c) differentiation antigens expressed by melanomas and melanocytes. This group includes tyrosinase (Wolfel et al., Eur. J. Immunol. 4:759 (1994) and Brichard et al., Eur. J. Immunol. 26:224 (1996)); gp 100 (Kang et
30 al., J. Immunol. 155:1343 (1995), Cox et al., Science 264:716 (1994), and Kawakami et al., J. Immunol. 155:3961 (1995)); gp75 (Wang et al., J. Exp. Med. 183:1131 (1996)), and Mart-1/MelanA (see US patent 5,620,886).

35

Of all these antigens, Mart-1/MelanA appears to be the best candidate for use in immunotherapy, this being for several reasons. Firstly, this antigen was identified on the basis of the CTL response, *in vivo*, of the

lymphocytes infiltrating the melanoma and not that, in vitro, of the peripheral blood cells, which would suggest greater relevance of this antigen in the natural response, in vivo, against melanoma (Kawakami
5 et al., J. Exp. Med. 180:347 (1994)). In addition, Mart-1/MelanA is expressed on all melanomas examined, which makes it a preferred target for intervention by immunotherapy. Finally, peptides derived from Mart-1/MelanA are capable of inducing a specific CTL
10 response in patients with melanoma expressing the HLA-A2 histocompatibility antigen (Rivoltini et al., J. Immunol. 154:2257 (1995); Valmori et al., J. Immunol. 160:1750 (1998)).

15 HLA-A2 is the most common allele expressed in Caucasians. The CTL epitopes of Mart-1/MelanA have been defined for this allele. The antigenic peptide recognized by the majority of human CTL lines comprises amino acids 27-35 AAGIGILTV (Kawakami et al., J. Exp.
20 Med. 180:347 (1994)). In addition, studies on the affinity of binding with HLA-A*0201 and recognition by CTL clones have demonstrated that the optimum peptide for these two functions is the 26-35 decapeptide EAAGIGILTV (Romero et al., J. Immunol. 159:2366
25 (1997)). However, it appears that these peptides are weakly immunogenic in vitro (Valmori et al., J. Immunol. 160:1750 (1998)) and in vivo (Jaeger et al., Int. J. Cancer 66:162 (1996)).

30 When comparing the amino acid sequence of the T epitopes of Mart-1/MelanA with the peptide motifs of A*0201 (Rammensee et al., Immunogenetics 41:178 (1995)), it appears that the 26-35 and 27-35 peptides have nondominant anchoring residues at position 2 and
35 therefore weakly bind the HLA-A*0201 molecule (Kawakami et al., J. Immunol. 154:3961 (1995)), which might explain their weak immunogenicity. The international patent application published under the number WO 98/58951 describes an analog to the 26-35 peptide,

in which the alanine at position 2 has been replaced with a leucine (sequence which will be named ELA of sequence SEQ ID No. 3).

5 The hELA peptide, used in the experiments below, is the subject of patent application WO 98/58951 which is the property of the Institut Ludwig de Recherche sur le Cancer [Ludwig Cancer Research Institute]. hELA is an analog of the 26-35 decapeptide (EAAGIGILTV) of
10 Melan-A/MART-1, which is a protein expressed on melanocytes and melanomas. Although the 26-35 decapeptide of Melan-A/MART-1 is capable of binding to the HLA-A0201 molecule (Romero et al., 1997, J. Immunol. 159, 2366-2374), it is weakly immunogenic
15 in vitro and in vivo (Valmori et al., 1998, J. Immunol. 160, 1750-1758). The hELA analog was generated by substituting the second amino acid of the 26-35 decapeptide of Melan-A/MART-1 (an alanine) with a leucine. The result of this substitution, which is
20 based on analysis of the residues required for anchoring the peptides to the HLA-A0201 molecule, is more effective recognition by the CTLs of patients with melanoma and better immunogenicity in vitro (Valmori et al., 1998, J. Immunol. 160, 1750-1758).
25 HLA-A*0201/K^b (A2/K^b) transgenic mice of the strain C57BL/6 x BDA/2 (Vitiello et al., 1991, J. Exp. Med., 173, 1007-1015) were used in this study to test ELA. The class I MHC molecule expressed in these mice is a chimeric molecule made from the α_1 and α_2 domains of
30 the human HLA-A0201 molecule (the most common allotype found) and from the α_3 domain of the murine K^b molecule.

The TRP-2 peptide of sequence SEQ ID No. 4 is an
35 octapeptide corresponding to amino acids 181-188 (VYDFFVWL) of tyrosinase-related protein 2 (TRP-2). TRP-2 is expressed in melanocytes and melanomas. It has been demonstrated that this antigen induces CTL responses which protect against melanoma in C57BL/6

(H-2K^b) mice (Bloom et al., 1997, J. Exp. Med. 185, 453-459).

5 A: Generation of anti-Melan-A and anti-TRP-2 CTLs after immunization with rP40 mixed with a peptide which is an analog to Melan-A or TRP-2

Experimental protocol

10 A2/Kb mice received, by subcutaneous injection at the base of the tail:

- 50 µg of ELA mixed with 3 or 300 µg of rP40;
- 50 µg of ELA covalently coupled to 300 µg of rP40.

15 C57BL/6 mice received, by subcutaneous injection into the base of the tail:

- 50 µg of the TRP-2 peptide (181-188) mixed with 300 µg of rP40.

20 Generation of cytotoxic effector cells

25 10 days after immunization, the mice are sacrificed and the lymphocytes from the draining lymph nodes are recovered in order to be stimulated, *in vitro*, with the relevant peptide.

30 These lymphocytes (4 to 5 × 10⁶) are cultured in a 24 well plate in DMEM plus 10 mM HEPES, 10% FCS and 50 µM β-2-mercaptoethanol, with 2 to 5 × 10⁵ EL-4 A2/Kb cells or EL4 cells which have been irradiated (10 kRads) and prepulsed for 1 h at 37°C with 1 µM of the relevant peptide. After two weekly stimulations, the cells are assayed for their cytotoxic activity.

35 Measurement of cytotoxic activity

The EL-4 A2/Kb cells or EL4 cells are incubated for 1 h with ⁵¹Cr in the presence or absence of the relevant peptide, washed and then coincubated with the effector cells at various ratios, in a 96-well plate in a volume

of 200 µl for 4 to 6 h at 37°C. The cells are then centrifuged and the ^{51}Cr release is measured in 100 µl of supernatant. The percentage of specific lysis is calculated as follows:

5 % specific lysis = (experimental release - spontaneous release) / (total release - spontaneous release) X 100.

Results

As shown in figures 1A to 1D, the immunization of mice
10 with an optimal dose of rP40 (300 µg) in a mixture with
hELA (figure 1B) or TRP-2 (figure 1D) induces a strong
specific CTL response. Such a response is also observed
after immunization with rP40 coupled to hELA
(figure 1C). On the other hand, the immunization with
15 the peptide alone or rP40 alone (results not shown) or
with the hELA peptide in a mixture with a suboptimal
dose of rP40 (3 µg) does not induce any CTL activity
(figure 1A). These results demonstrate that the rP40
molecule mixed with or coupled to immunogenic peptides
20 makes it possible to induce a specific CTL response
in vivo, this being without the addition of adjuvant.

B: Generation of anti-Melan-A CTLs after immunization
with rP40 mixed with a peptide which is an analog to
25 Melan-A, compared to a standard immunization protocol

Experimental protocol

A2/Kb mice received:

- 50 µl of IFA (incomplete Freud's adjuvant) by
30 subcutaneous injection at the base of the tail, then,
3 weeks later, 50 µg of hELA in the presence of 50 µg
of a helper-T p30 peptide derived from Tetanus Toxoid
(TT) (Panina-Bordignon et al., Eur. J. Immunol., 1989,
19, 2237) adjuvanted with IFA. This protocol has been
35 described for generating anti-peptide CTLs (Valmori
et al., Eur. J. Immunol., 1994, 24, 1458) and is used
as a positive control.
- 50 µg of hELA alone or 300 µg of rP40 mixed with
or coupled to 50 µg of hELA.

Generation of cytotoxic effector cells

10 days after the final immunization, the mice are
5 sacrificed and the lymphocytes from the draining lymph
nodes are recovered in order to be stimulated,
in vitro, with the relevant peptide.

These lymphocytes (4 to 5×10^6) are cultured in a
10 24-well plate in DMEM plus 10 mM HEPES, 10% FCS and
 50 μ M β -2-mercaptoethanol, with 2 to 5×10^5 EL-4 A2/Kb
cells (murine cells transfected with the HLA-A* 0201/Kb
gene) which have been irradiated (10 kRads) and
15 prepulsed for 1 h at 37°C with 1 μ M of the relevant
peptide.

After one, two or three weekly stimulations, the cells
are assayed for their cytotoxic activity.
20 The cytotoxic activity is measured according to the
method described above.

Results

25 After immunization with nonadjuvanted rP40 coupled to
hELA, an anti-hELA CTL activity comparable to that
observed after immunization with hELA + P30/IFA is
measured (cf. figures 2C and 2D). Similarly, the rP40 +
30 hELA peptide mixture, itself also nonadjuvanted,
generates CTLs in a way which is similar to that
obtained with a conventional protocol for generating
CTLs (cf. figures 2B and 2D).

No CTL activity was detected after immunization with
35 the peptide alone (cf. figure 2A) or the rP40 protein
alone (not shown), regardless of the day on which the
effector cells were stimulated.

Example 5: Antitumor effect of the immunization with a mixture of rP40 and of a peptide expressed by a mouse melanoma

5 In order to evaluate the capacity of rP40 to generate an antitumor CTL response, the capacity of the rP40 protein to induce a CTL response directed against the peptide of sequence SEQ ID No. 4 (VYDFFVWL) was tested.
10 The peptide of sequence SEQ ID No. 4 (VYDFFVWL) is derived from Tyrosinase Related Protein 2 (TRP-2) which is expressed by the B16F10 melanoma derived from C57BL/6 mice. This peptide is immunogenic in this strain. The growth of the B16F10 cells implanted into C57BL/6 mice which were or were not immunized with a
15 mixture of rP40 and of the TRP-2 peptide was then tested.

Experimental protocol

20 For generating an anti-TRP-2 peptide CTL response, a protocol identical to that described in example 4 was used, except that, on this occasion, C57BL/6 mice were used.
25 For the protection experiments, C57BL/6 mice received 2×10^3 cells of the B16F10 autologous melanoma, by subcutaneous (s.c.) injection into the flank. Simultaneously, or 4 days later, some of these mice were immunized subcutaneously (at the base of the tail)
30 with 50 µg of the TRP-2 peptide mixed with 300 µg of rP40. The growth of the tumor was then measured at regular intervals.

Results

35 As shown in figure 3A, the immunization with a mixture of TRP-2 peptide and of the RP40 protein is capable of generating a specific CTL response to this peptide, which confirms the results obtained with the hELA

protein (described in example 4). In addition, this CTL response is associated with inhibition of the growth of the B16F10 melanoma (figures 3B, 3C and 3D). It is of value to note that this protection is significant not
5 only when the immunization with the TRP-2 peptide + rP40 is carried out simultaneously with the implantation of the tumor (figures 3b and 3D), but also when carried out 4 days after the implantation (figure 3C).

10

These results clearly show the therapeutic effect of the use of an enterobacterium OmpA protein, such as the *K. pneumoniae* OmpA protein, combined with an antigenic tumor peptide, in order to induce a specific CTL-type
15 response which is effective in preventing or treating cancer, such as melanomas.

20 Example 6: Generation of anti-OVA CTLs after immunization with rP40 coupled to the p257-264 Ova peptide

The p257-264 Ova peptide is an octapeptide corresponding to the fragment of the ovalbumin consensus sequence which is between the amino acids at
25 position 257 to 264 of the ovalbumin sequence (ends included). Ovalbumin is used as a peptide which protects against tumor cells expressing ovalbumin.

Experimental protocol

30

C57BL/6 mice received, by subcutaneous injection into the base of the tail, 200 µg of P40-Ova (□), of Ova-coupled beads (○), of solubilized Ova (■), of Ova-BS³ (□) (BS³ for bis(succinimidyl) suberate), of P40 (←) or of DT-Ova (●) (DT for Diphtheria Toxoid).

Generation of cytotoxic effector cells

7 days after immunization, the mice are sacrificed and the spleens are recovered. The spleen cells (4×10^7)
5 are cultured in flasks, in DMEM with 1.5×10^6 irradiated (4kRads) E.G7 cells.

Measurement of the cytotoxic activity

10 The EL4 thymoma cells which were pulsed or not pulsed with the OVA peptide or transfected with the ova gene (E.G7 line) are incubated with ^{51}Cr at 37°C and cultured with the effector cells obtained above.

15 The percentage of specific lysis is calculated as described in example 4A.

Results

20 As shown in figures 4A to 4C, the immunization of mice with the rP40 protein coupled to or mixed with the OVA peptide induces a strong specific CTL response. This response is similar to that observed after immunization with the positive control, namely the ovalbumin-coupled
25 beads (see figures 4A and 4B). On the other hand, the immunization with soluble ovalbumin, ova-BS³ and DT-Ova is not effective. These results demonstrate that the rP40 module coupled to an immunogenic peptide makes it possible to induce a specific CTL response *in vivo*,
30 this being without the addition of adjuvant.

CLAIMS

1. The use of an enterobacterium OmpA protein, or of a fragment thereof, for preparing a pharmaceutical composition intended to generate or increase a cytotoxic T response against an infectious agent or a tumor cell.
5
2. The use as claimed in claim 1, characterized in that said pharmaceutical composition also comprises, combined with said enterobacterium OmpA protein, an antigen or a hapten specific for said infectious agent or for said tumor cell.
10
3. The use as claimed in either of claims 1 and 2, characterized in that said infectious agent is a viral particle, a bacterium or a parasite.
15
4. The use as claimed in one of claims 1 to 3, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said enterobacterium.
20
5. The use as claimed in one of claims 1 to 3, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained via the recombinant route.
25
6. The use as claimed in one of claims 1 to 5, characterized in that said enterobacterium is Klebsiella pneumoniae.
30
7. The use as claimed in claim 6, characterized in that the amino acid sequence of said OmpA protein, or a fragment thereof, comprises:
 - a) the amino acid sequence of sequence SEQ ID No. 2;
35

b) the amino acid sequence of a sequence having at least 80% homology with the sequence SEQ ID No. 2; or

5 c) the amino acid sequence of a fragment of at least 5 amino acids of a sequence as defined in a).

10 8. The use as claimed in one of claims 2 to 7, characterized in that said antigen or hapten is chosen from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing the CTL response against said infectious agent or said tumor cell.

15 9. The use as claimed in one of claims 2 to 8, characterized in that said antigen or hapten is coupled to or mixed with said OmpA protein or a fragment thereof.

20 10. The use as claimed in claim 9, characterized in that said antigen or hapten is coupled, by covalent attachment, with said OmpA protein or a fragment thereof.

25 11. The use as claimed in claim 10, characterized in that the coupling by covalent attachment is coupling produced by chemical synthesis.

30 12. The use as claimed in claim 11, characterized in that one or more attachment elements is(are) introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate the chemical coupling.

35 13. The use as claimed in claim 12, characterized in that said attachment element introduced is an amino acid.

14. The use as claimed in claim 10, characterized in
that the coupling between said antigen or hapten
and said OmpA protein, or a fragment thereof, is
produced by genetic recombination, when said
5 antigen or hapten is peptide in nature.

15. The use as claimed in claim 14, characterized in
that the pharmaceutical composition comprises a
nucleic acid construct encoding said hybrid
10 protein.

16. The use as claimed in claim 15, characterized in
that said nucleic acid construct is contained in a
vector, or in a transformed host cell capable of
expressing said hybrid protein.
15

17. The use as claimed in one of claims 1 to 16, for
preparing a pharmaceutical composition intended to
eliminate infectious agents or inhibit tumor
growth.
20

18. The use as claimed in one of claims 1 to 17, for
preparing a pharmaceutical composition intended to
prevent or treat infectious diseases comprising
viral, bacterial, fungal and parasitic infections.
25

19. The use as claimed in one of claims 1 to 17, for
preparing a pharmaceutical composition intended to
prevent or treat cancers.
30

20. The use as claimed in claim 19, for preparing a
pharmaceutical composition intended to prevent or
treat cancers associated with a tumor antigen.

35 21. The use as claimed in claims 19 and 20, for
preparing a pharmaceutical composition intended to
prevent melanomas.

22. The use as claimed in one of claims 1 to 21, characterized in that said pharmaceutical composition is vehicled in a form which makes it possible to improve its stability and/or its immunogenicity.

5

23. The use as claimed in claim 22, characterized in that said vehicle is a liposome, a viral vector containing a nucleic acid construct encoding said OmpA protein, or a fragment thereof, said antigen or hapten, or said hybrid protein, or a transformed host cell capable of expressing said OmpA protein, or a fragment thereof, said antigen or hapten, or said hybrid protein.

10

15

24. The use as claimed in one of claims 15, 16 and 23, characterized in that said nucleic acid construct, or the nucleic acid construct contained in said vector or said transformed host cell, comprises a nucleic acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides of the sequence SEQ ID No. 1, or a sequence having at least 80% homology with one of said sequences.

20

25

25. A pharmaceutical composition, characterized in that it comprises, in a pharmaceutically acceptable medium, at least one enterobacterium OmpA protein, or a fragment thereof, combined, by mixing or by coupling, with at least one antigen or one hapten associated with or specific for a tumor cell.

30

35

26. The composition as claimed in claim 25, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained using a method of extraction from a culture of said enterobacterium.

27. The composition as claimed in claim 25, characterized in that said enterobacterium OmpA protein, or a fragment thereof, is obtained via the recombinant route.

5

28. The composition as claimed in one of claims 25 to 27, characterized in that said enterobacterium is Klebsiella pneumoniae.

10 29. The composition as claimed in claim 28, characterized in that the amino acid sequence of said OmpA protein, or a fragment thereof, comprises:

15 a) the amino acid sequence of sequence SEQ ID No. 2;
b) the amino acid sequence of a sequence having at least 80% homology with the sequence SEQ ID No. 2; or
c) the amino acid sequence of a fragment of at least 5 amino acids of a sequence as defined in a).

20 30. The composition as claimed in one of claims 25 to 29, characterized in that said antigen or hapten is chosen from peptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids or any compound capable of specifically directing a CTL response against said tumor cell.

25 31. The composition as claimed in one of claims 25 to 30, characterized in that said antigen or hapten is coupled, by covalent attachment, with said OmpA protein or a fragment thereof.

30 32. The composition as claimed in claim 31, characterized in that the coupling by covalent attachment is coupling produced by chemical synthesis.

33. The composition as claimed in claim 32, characterized in that one or more attachment elements is(are) introduced into said OmpA protein, or a fragment thereof, and/or into said antigen or hapten, in order to facilitate the chemical coupling.

5

34. The composition as claimed in claim 33, characterized in that said attachment element introduced is an amino acid.

10

35. The composition as claimed in claim 31, characterized in that the coupling between said antigen or hapten and said OmpA protein, or a fragment thereof, is produced by genetic recombination, when said antigen or hapten is peptide in nature.

15

36. The composition as claimed in claim 35, characterized in that the pharmaceutical composition comprises a nucleic acid construct encoding the hybrid protein obtained after said coupling.

20

25 37. The composition as claimed in claim 36, characterized in that said nucleic acid construct is contained in a vector, or in a transformed host cell capable of expressing said hybrid protein.

30

38. The composition as claimed in either of claims 36 and 37, characterized in that said nucleic acid construct comprises a nucleic acid sequence chosen from the sequence SEQ ID No. 1, a fragment thereof having at least 15 consecutive nucleotides of the sequence SEQ ID No. 1, or a sequence having at least 80% homology with the sequence SEQ ID No. 1.

35

39. The composition as claimed in one of claims 25 to 38, characterized in that said pharmaceutical

composition is vehicled in a form which makes it possible to improve its stability and/or its immunogenicity.

5 40. The composition as claimed in claim 39,
characterized in that said vehicle is a liposome,
a viral vector containing a nucleic acid construct
encoding said OmpA protein, or a fragment thereof,
said antigen or hapten, or said hybrid protein, or
10 a transformed host cell capable of expressing said
OmpA protein, or a fragment thereof, said antigen
or hapten, or said hybrid protein.

15 41. The composition as claimed in one of claims 25 to
40, characterized in that said pharmaceutically
acceptable medium consists of water, of an aqueous
saline solution or of an aqueous solution based on
dextrose and/or on glycerol.

20 42. The composition as claimed in one of claims 25 to
41, characterized in that said composition also
contains a detergent.

25 43. The composition as claimed in one of claims 25 to
42, without any other adjuvant for inducing a CTL
response.

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531 Rec'd PCT 16 AUG 2001

ABSTRACT OF THE DISCLOSURE

The invention concerns the use of an enterobacterium OmpA membrane protein, in particular of *Klebsiella pneumoniae* associated with an antigen or a hapten for preparing a pharmaceutical composition for generating or enhancing a cytotoxic T response directed against an infectious or tumor cell. The invention also concerns the use of the compounds for preventing and treating infection or cancer, in particular cancers associated with a tumoral antigen such as melanoma, and pharmaceutical compositions comprising some of the compounds.

09/913772 531 Rec'd PCT 16 AUG 2001

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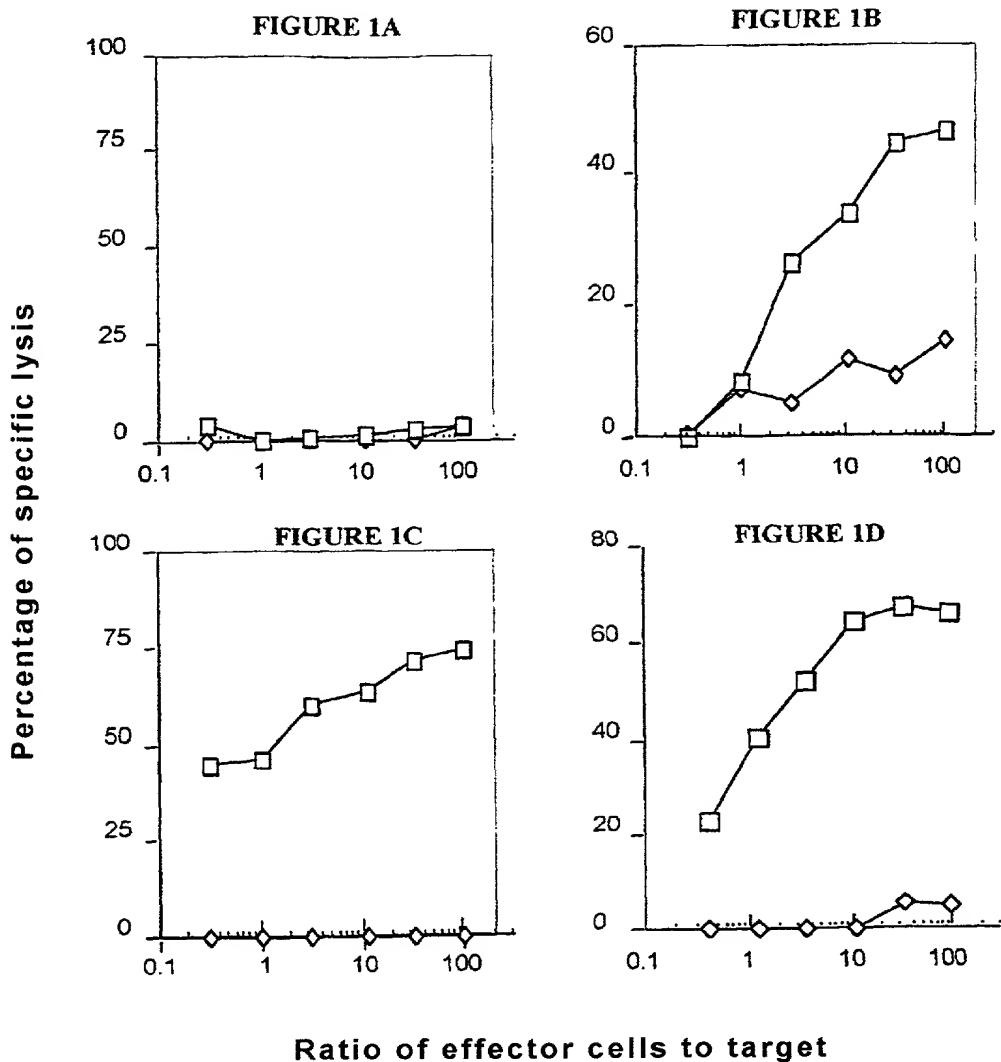
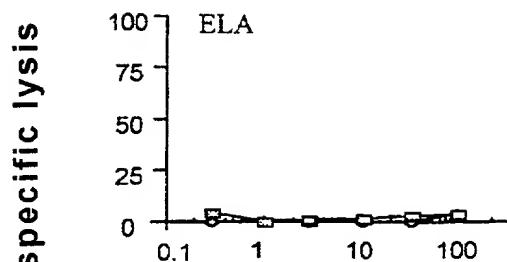
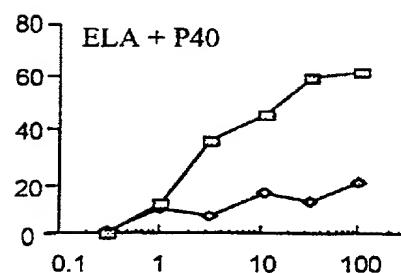
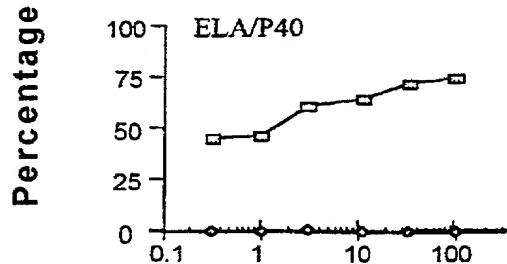
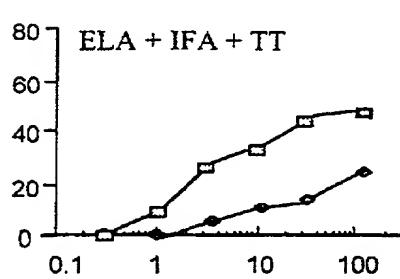


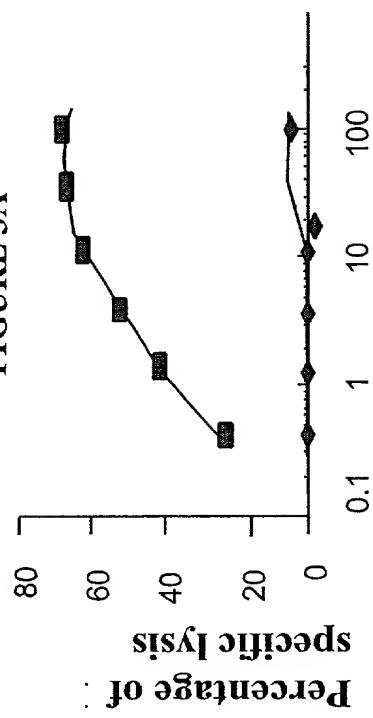
FIGURE 2A**FIGURE 2B****FIGURE 2C****FIGURE 2D**

Percentage of specific lysis

Ratio of effector cells to target cells

09/913772

FIGURE 3A



Ratio of effector cells to target cells

FIGURE 3B

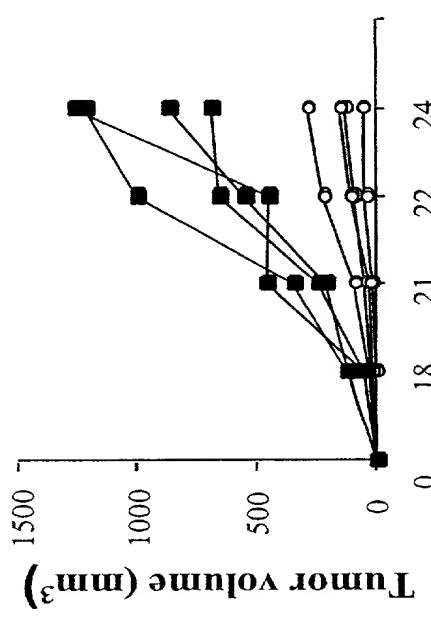


FIGURE 3C

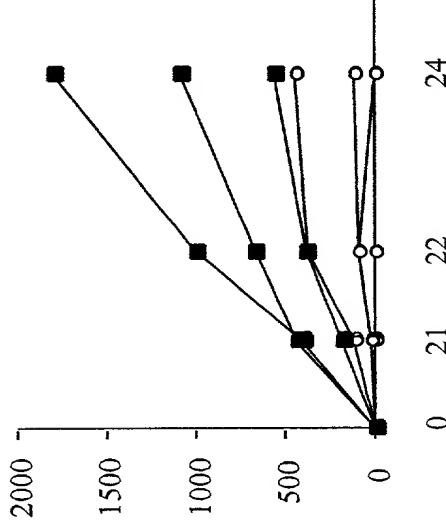
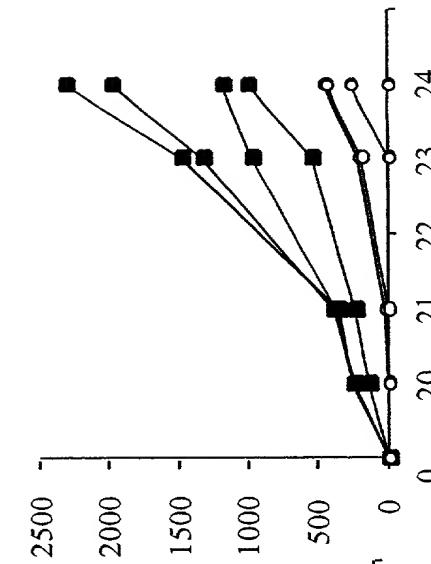
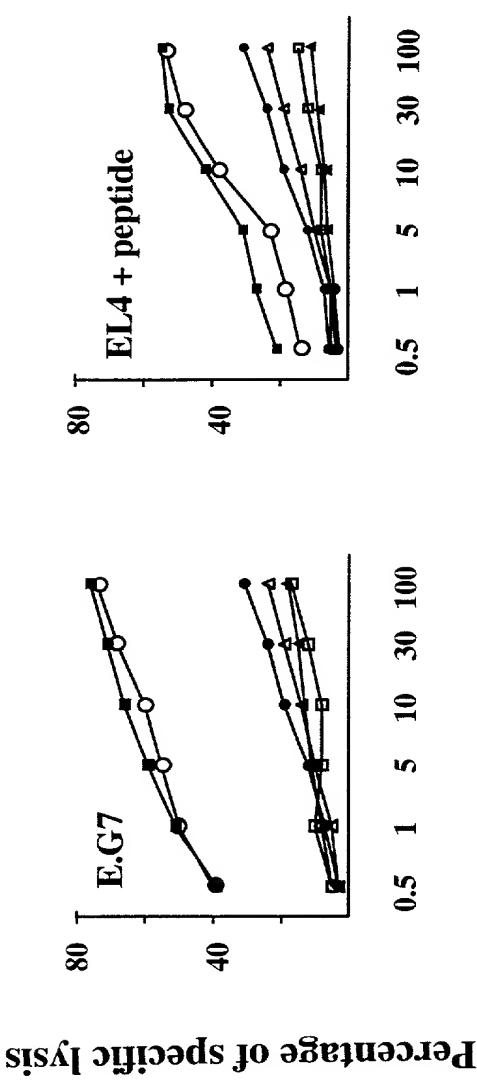
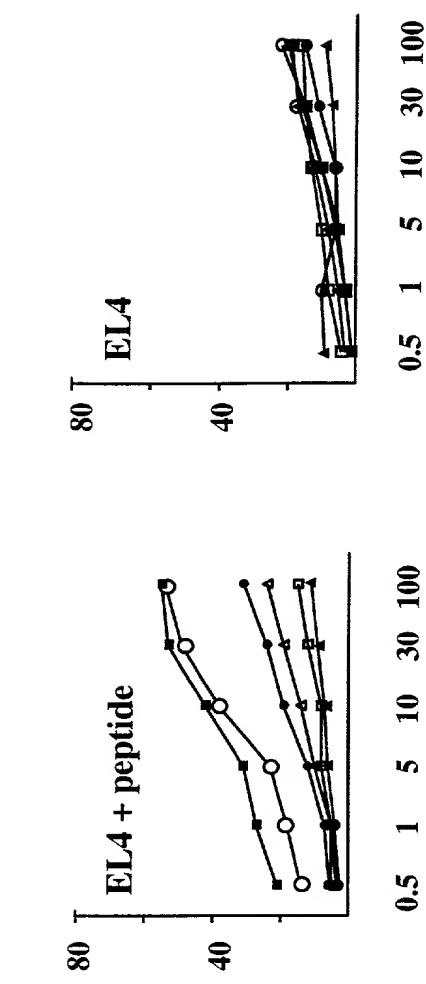
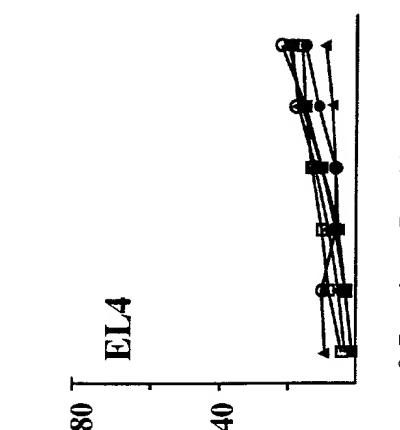


FIGURE 3D

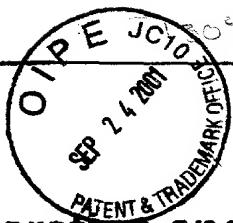


Number of days after implantation

FIGURE 4A**FIGURE 4B****FIGURE 4C**

Ratio of effector cells to target cells

Percentage of specific lysis



457(1)

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Declaration and Power of Attorney For Patent Application

English Language Declaration

- As a below named inventor, I hereby declare that:
- My residence, post office address and citizenship are as stated below next to my name.
- I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**USE OF AN ENTEROBACTERIUM OmpA PROTEIN COMBINED WITH AN ANTIGEN, FOR
GENERATING AN ANTIVIRAL, ANTI PARASITIC OR ANTITUMOR CYTOTOXIC RESPONSE**

the specification of which

(check one)

is attached hereto.

was filed on AUGUST 10, 2001 as United States Application No. or PCT International

Application Number _____

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

<u>9901917</u> (Number)	<u>FRANCE</u> (Country)	<u>17 FEBRUARY 1999</u> (Day/Month/Year Filed)	<input type="checkbox"/>
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I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

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(Application Serial No.)

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I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

FR00/00393

17 FEBRUARY 2000

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(Application Serial No.)

(Filing Date)

(Status)

(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)

(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)

(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

G. Patrick Sage #37,710

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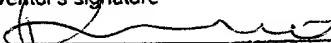
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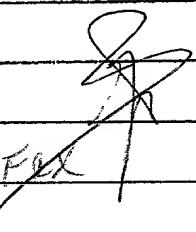
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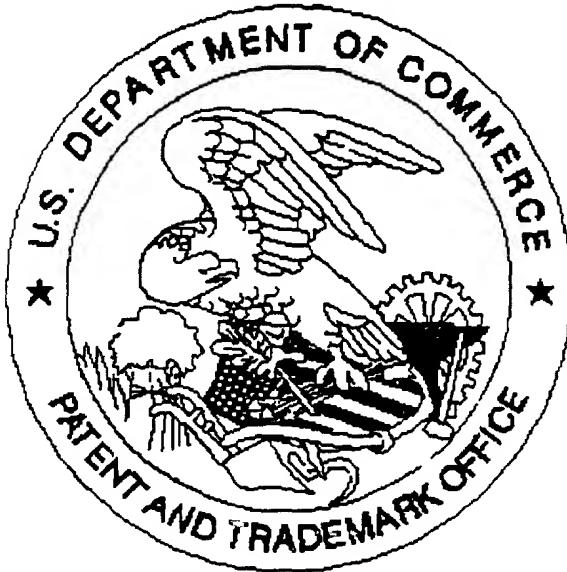
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616-382-0030

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